Inhibition of the covalent binding reaction of complement component C4 by penicillamine, an anti-rheumatic agent

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D(–)-Penicillamine [D(–)- $\beta\beta$ -dimethylcysteine] is an anti-arthritis drug, but its use is limited by adverse side effects, which include problems in immune-complex clearance. Complement is important as a source of inflammatory mediators in rheumatoid arthritis and is also involved in immune-complex clearance. Thus inhibition of the complement cascade would be likely to contribute to both the therapeutic and the toxic effects of penicillamine. It is shown that penicillamine and cysteine are potent inhibitors of the covalent binding of activated complement component C4 to immune complexes. [35 S]Cysteine itself becomes covalently bound to C4b through the thioester site. Penicillamine and cysteine are more reactive with the C4A isotype than with the C4B isotype of the HLA class III protein C4. The limited amino acid sequence differences between C4A and C4B include a cysteine/serine interchange, and it is suggested that the cysteine residue in C4A contributes to the increased rate of reaction of C4A with the α -amino- β -thiol compounds.

INTRODUCTION

During activation of the classical complement cascade by antibody–antigen complexes, component C4 is cleaved by the proteinase C1s, which is bound to the immune complex. C1s cleaves C4 to C4a, an anaphylatoxin, and the major fragment C4b, which then, for a short time (less than 1 ms), has the ability to bind covalently to the immune complex by an acyl transfer reaction. C4b bound to the complex then forms part of the enzyme C4b2a, which cleaves C3 to C3a, an anaphylatoxin, and C3b, which, like C4b, has the ability to bind covalently to the complex and to participate in generation of C5a, a potent anaphylatoxin and chemotactic factor (for review see Campbell *et al.*, 1988). C3b deposition also prevents precipitation of immune complexes (Schifferli *et al.*, 1980), and patients with deficiencies of C1, C2 and C4 are at increased risk of disorders of immune-complex handling in which immune complexes become deposited at inappropriate sites in the body, particularly in the small blood vessels and kidney glomeruli (Lachmann, 1984). Complement-derived activation fragments, particularly C5a (Wedmore & Williams, 1981), are likely to be important inflammatory mediators in rheumatoid arthritis (Moxley & Ruddy, 1987).

D(–)-Penicillamine [D(–)- $\beta\beta$ -dimethylcysteine] has been used as an anti-rheumatic agent, but its mechanism of action is not clearly established, although reduction of disulphide bridges in Rheumatoid Factor (Wernick *et al.*, 1983) and inhibition of collagen cross-linking (Nimni *et al.*, 1972) have been suggested. One major problem with penicillamine as an anti-rheumatic drug is that there is a high incidence of patients suffering adverse side effects, which include problems in clearance of immune complexes with resulting proteinuria and glomerulo-

nephritis (Andrews *et al.*, 1973; Davison *et al.*, 1977; Kean *et al.*, 1980). Drugs that induce this type of side effect have been shown to inhibit complement component C4 (Sim *et al.*, 1984). Previous investigators have commented that the amount of complement component C3 deposited in the joints of patients receiving penicillamine was greatly diminished (Mellbye & Munthe, 1977), and therefore we investigated the effect of penicillamine on C4 activation.

C4 is encoded at two highly polymorphic loci, C4A and C4B, within the class III region of the major histocompatibility complex. Each C4 isotype, C4A and C4B, exists in multiple allelic forms, e.g. C4A3 and C4B1, which are the most commonly found allotypes. Normal individuals have four C4 genes, two for C4A allotypes and two for C4B allotypes. Null alleles for C4A or C4B with no C4 protein product are also found in certain individuals (Mauff *et al.*, 1983).

Although C4A and C4B isotypes share many activities, the C4A allotypes bind covalently more readily to complement-activating surfaces rich in amino groups, as opposed to C4B allotypes, which bind more readily to hydroxy groups on the complement-activating surface (Isenman & Young, 1984; Law *et al.*, 1984). In view of the different spectra of reactivities of C4A and C4B isotypes, the effect of penicillamine on C4A3 and on C4B1 has now been investigated.

MATERIALS AND METHODS

Haemolytic assays of C4

Isolated C4A3 and C4B1 were prepared as previously described (Dodds *et al.*, 1985). Haemolytic activity was

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determined in two ways: (a) C4 was serially diluted in buffer containing a constant concentration of penicillamine or cysteine, and (b) C4 was pre-incubated in the presence of a potential inhibitor and then the C4 and the potential inhibitor were serially diluted simultaneously.

(a) C4A or C4B was serially diluted in 100 μ l of DGVB²⁺ (140 mM-glucose/71 mM-NaCl/0.5 mM-MgCl₂/0.15 mM-CaCl₂/2.5 mM-sodium 5,5-diethylbarbiturate buffer, pH 7.5, containing 1 g of gelatine/l) alone or of DGVB²⁺ containing penicillamine or cysteine added from a 100 mM stock solution, pH 7.5, prepared daily. Sheep erythrocytes sensitized with antibody and guinea-pig C1 (EAC1) (100 μ l of 10⁸ cells/ml of DGVB²⁺) were added and incubated at 37 °C for 1 h. Then 1 ml of ice-cold buffer (116 mM-glucose/59 mM-NaCl/70 mM-MgCl₂/100 mM-EGTA/2 mM-sodium 5,5-diethylbarbiturate buffer, pH 7.5) was added, cells were centrifuged (2000 *g* for 10 min) and the supernatant was discarded to remove the inhibitor. The cell button was resuspended in 100 μ l of DGVB²⁺, and 100 μ l of C4-deficient guinea-pig serum (diluted 1:75 with DGVB²⁺) was added. Lysis was determined in the supernatant after incubation for 1 h at 37 °C (Whaley, 1985). The reciprocal dilution of C4 is plotted against $-\ln(1-Y)$, where *Y* represents percentage lysis (Whaley, 1985). The dilution where *Y* = 63% was determined at $-\ln(1-Y) = 1$ and is the dilution of C4 that was used to determine percentage inhibition in the presence of penicillamine. Zero percentage inhibition was determined in the absence of penicillamine.

(b) C4A (110 μ g) was incubated (at 37 °C for 20 min) in a total volume of 25 μ l of DGVB²⁺ alone or DGVB²⁺ containing 5 mM-penicillamine. C4 or C4 plus penicillamine was then serially diluted in DGVB²⁺ and the haemolytic activity was measured as described in (a).

Covalent binding of [³⁵S]cysteine to C4

C4A3 or C4B1 (Dodds *et al.*, 1985) was activated by pure human C1s (Law *et al.*, 1984) in the presence of 50 μ l of [³⁵S]cysteine (50 mCi/mmol). Samples were run on SDS/polyacrylamide-gel electrophoresis with 7.5% polyacrylamide gels with or without reduction with dithiothreitol, and the radioactivities bound to the whole C4 (non-reduced) or to the α' -chain (reduced) were determined (Dodds *et al.*, 1985) with the use of NCS tissue solubilizer to recover radioactive material from the gel. Radioactivity associated with the β - and γ -chains was negligible.

The rate of reaction of C4 with radiolabelled nucleophiles was determined by incubation of C4A3 or C4B1 with human C1s as above but in the presence of [³H]glycine (200 mCi/mmol), [³H]glycerol (200 mCi/mmol) or [³⁵S]cysteine as above in 125 mM-NaCl/0.5 mM-EDTA/10 mM-potassium phosphate buffer, pH 7.4. The amount of radioactivity that bound to the reactive-site thioester in the α' -chain was determined after SDS/polyacrylamide-gel electrophoresis run under reducing conditions. The amount of reactive protein was determined by direct incorporation of [³H]methylamine (200 mCi/mmol) into the intact thioester bond in the absence of C1s (Dodds *et al.*, 1985). The percentage of C4 molecules that bound the nucleophiles was calculated, as were the reaction rates (Dodds *et al.*, 1985). In the calculated reaction rate (k'/k_0), k' is the second-order reaction rate of C4 with the nucleophile and k_0 is the first-order rate of hydrolysis of the activated thioester.

RESULTS

Effects of penicillamine and cysteine on C4 haemolytic activity

Penicillamine inhibits the haemolytic activity of C4 when the drug is present during the assay (Fig. 1), and is a more potent inhibitor of C4A than of C4B (Fig. 1). If C4 is pre-incubated with penicillamine before determination of C4 activity, no inhibition is observed over the same concentration range (Fig. 2). The lack of effect of penicillamine on C4 haemolytic activity when assayed in this way (Fig. 2) is in agreement with results obtained by others who have investigated the effect of penicillamine using a similar method to that shown in Fig. 2 (Mellbye & Munthe, 1977; von Zabern & Nolte, 1987), and accounts for the lack of any decrease in complement activity measured in the plasma of individuals taking penicillamine (Mellbye & Munthe, 1977). The plasma

Fig. 1. (a) Penicillamine inhibition of the haemolytic activity of human C4A and human C4B with the drug present during activation of C4, and (b) the effect of cysteine on the haemolytic activity of human C4A3 and C4B1

In (a) the effect of penicillamine was determined on C4 haemolytic activity as described in the Materials and methods section. A typical plot of the reciprocal of the dilution of C4 against $-\ln(1-Y)$ is shown in the inset, where C4A was diluted in DGVB²⁺ alone (■) or in DGVB²⁺ containing 500 μ M-penicillamine (●) or in DGVB²⁺ containing 1 mM-penicillamine (▲). The average $\pm$ S.D. for 11 determinations is shown for the inhibition of lysis plotted against the concentration of penicillamine. For (b), see the text for experimental details.

Fig. 2. Penicillamine does not inhibit C4 when C4 is incubated with the drug before activation

The haemolytic activity of C4 was determined by the assay method (b), in which C4A in DGVB²⁺ (■) or C4A in DGVB²⁺ plus 5 mM-penicillamine (▨) was serially diluted in DGVB²⁺ over the range 1:500 to 1:256000, and the percentage lysis is shown.

Table 1. Covalent binding of [³⁵S]cysteine to isolated C4A3 and C4B1

For experimental details see the text.

	Radioactivity bound (c.p.m.)	
	Non-reduced	Reduced
C4A3	759	885
C4B1	436	524

has to be diluted around 1000-fold to quantify complement activity, and this dilutes the drug also.

Cysteine was found to inhibit C4 activity in a very similar manner to penicillamine (Fig. 1b). It inhibits the activity of C4A more than C4B activity when present during the haemolytic assay, but it does not inhibit C4 if present only before activation. Neither penicillamine nor cysteine at up to 20 mM disrupts the interchain disulphide bridges in C4, nor do they reduce the rate of cleavage of C4 by C1s (results not shown).

D(-)-Penicillamine, L(+)-penicillamine and the racemic mixture behave in the same manner. The D isomer is used as a drug because the L form is too toxic as a therapeutic agent.

Covalent binding of [³⁵S]cysteine to C4 on activation

C4 was incubated with C1s in the presence of [³⁵S]cysteine since radioactive penicillamine was not available. Cysteine binds covalently to C4 and is not released by reduction of C4 with dithiothreitol (Table 1). The slight increase in radioactivity detected on reduction is likely to be due to greater ease of solubilization of the smaller α' -chain with tissue solubilizer for liquid-scintillation counting compared with the whole C4 molecule. Therefore cysteine, and by inference penicillamine, is not bound to C4 by a disulphide bridge. Since an

Scheme 1. Scheme for reaction of penicillamine and cysteine with the thioester site in C4

The thioester site is exposed on activation of C4 to produce short-lived 'nascent' C4b, and the amino thiol compound reacts with the exposed carbonyl group to form a thiazolidine ring. R = CH₃ in penicillamine and R = H in cysteine.

Table 2. Reaction rates of isolated C4A3 and C4B1 with nucleophiles

For experimental details see the text.

Nucleophile	Concn. (μ M)	Percentage bound		Reaction rate [k'/k_0 (M^{-1})]	
		C4A	C4B	C4A	C4B
Cysteine	100	98	62	—	16300
	10	80	18	400000	22000
	1	31	4	450000	—
Glycine	2500	100	19	—	93.8
	50	48	0.9	19000	—
Glycerol	10000	0.8	11	0.8	12.7

active-site thioester in C4 is known to be exposed on activation by C1s (Janatova, 1983), it therefore seems very likely that cysteine binds to C4 through this thioester carbonyl group. Interaction of amino thiol compounds with carbonyl groups has been well documented (Ratner & Clarke, 1937; Abbott & Martell, 1970; Friedman, 1977), and [³⁵S]cysteine binds covalently to the chain of activated C4 that is the chain containing the active-site thioester (Table 1).

The mechanism proposed for interaction of the thiol compounds with C4 is shown in Scheme 1. It is known that C4A is more reactive with amino compounds than is C4B, whereas C4B is more reactive with hydroxy nucleophiles (Law *et al.*, 1984; Isenman & Young, 1984). Although penicillamine and cysteine do each have an amino group, the rate of reaction of cysteine with C4A is 20 times greater than the rate of reaction of glycine, an amino nucleophile. C4B also reacts more rapidly with cysteine than with glycine. This is illustrated by the values of k'/k_0 in Table 2. Therefore the interaction of cysteine, and by inference penicillamine, with C4 is likely to be due to initial interaction of the thiol group with the carbonyl group of the thioester in C4, as shown in Scheme 1. The thiol group has been reported to be 7 times more reactive as a nucleophile than the amino group in penicillamine (Friedman, 1977), which supports the reaction sequence shown in Scheme 1.

DISCUSSION

The difference in reactivity with amino and hydroxy groups between C4A and C4B isotypes has been identified as residing in four amino acid differences between C4A and C4B isotypes. These occur in a block of six amino acid residues some 110 residues C-terminal of the reactive thioester group (Yu *et al.*, 1986). This group of four amino acids must be close to the exposed thioester group in the tertiary structure of the protein, since these changes correlate with the difference in reactivity towards amino and hydroxy compounds of the thioester group in C4A and C4B (Dodds *et al.*, 1985). One of the four changes between C4A and C4B involves a cysteine/serine interchange. The cysteine residue in C4A is likely to play a part in promoting the increased reactivity of C4A with penicillamine, perhaps through disulphide-bridge formation with the drug. In model studies, disulphide-bridged penicillamine dimers have been shown to undergo thiazolidine-ring formation with a carbonyl group (Abbott & Martell, 1970).

The major amplification step of the complement system involves C3, the central component. C3 has an active-site thioester and is activated by an analogous mechanism to C4 (Janatova, 1983). However, in the classical complement pathway, activation of C4 is an essential step to allow formation of the enzyme that in turn activates C3. So inhibition of C4 by penicillamine could prevent the amplification of C3 fixation following activation of the classical complement pathway by antibody-antigen complexes, leading to decreased C3 deposition in rheumatic joints, as has been observed in patients treated with penicillamine (Mellbye & Munthe, 1977).

C4 is an HLA class III antigen with two polymorphic loci, A and B. The difference in reactivity of the gene products with increased reaction of C4A with nitrogen nucleophiles (Law *et al.*, 1984; Isenman & Young, 1984) is such that the C4A isotype is likely to be the more important in immune-complex clearance involving non-cellular antigens (Law *et al.*, 1984). Recently anti-histone antibodies have been described as being diagnostic of drug-induced immune-complex disease in patients receiving the anti-arrhythmic agent procainamide (Totteritis *et al.*, 1988). Such complexes with histone as antigen are likely to be more reactive with C4A than C4B owing to the high concentration of amino groups in histones as potential covalent binding sites for the thioester carbonyl

group in C4A. Although rheumatoid arthritis is not associated with any particular C4 type (Grennan & Dyer, 1988) within the subpopulation of patients who develop toxic effects to penicillamine, there is an increased incidence of HLA-DR3 (Emery *et al.*, 1984) and this is in turn in linkage disequilibrium with a null (non-expressed) allele at the C4A locus (Awdeh *et al.*, 1983). There has been no report of C4 types of individuals showing penicillamine toxicity.

The kidney-associated side effects, which involve immune-complex deposition, are found on long-term treatment with penicillamine, and in this respect resemble drug-induced systemic lupus erythematosus (Harpey, 1973). Null alleles of C4 (Fielder *et al.*, 1983) and lack of other early complement components (Lachmann, 1984) are associated with idiopathic systemic lupus erythematosus. It has been suggested that inhibition of C4, particularly C4A (Sim & Law, 1985), by hydralazine leads to deposition of immune complexes and is important in drug-induced systemic lupus erythematosus (Sim *et al.*, 1984). In rheumatoid-arthritic patients with C4A null it would seem likely that inhibition of C4 by penicillamine in these patients could promote deposition of immune complexes in the kidney and subsequent damage. Plasma concentrations of 60 μM -penicillamine have been measured (Muijsers *et al.*, 1979), and the drug is likely to be present mainly in the reduced thiol form (Bourke *et al.*, 1984). Inhibition of C4A covalent binding would be observed at this concentration. In addition to C4 type, other genetic factors are likely to contribute to the development of toxicity, since there is an increased incidence of patients showing genetically determined impaired oxidation of penicillamine to the sulphoxide form (Emery *et al.*, 1984) among those who show an adverse reaction. These toxic effects are also observed in patients taking penicillamine for its Cu^{2+} -ion-chelating properties, as in Wilson's disease (Walshe & Golding, 1977), although no information has been published on the histocompatibility type of these patients.

It is not clear how the other less frequent types of toxic side effects that have been observed with penicillamine, namely myasthenia gravis and leucocyte disorders (Dawkins *et al.*, 1983), could be accounted for by the effects of penicillamine on C4, but the major kidney-associated side effects on long-term usage and the therapeutic action are both likely to reside in inhibition of covalent binding of C4A on activation.

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